



Research Article

DNA barcoding of some Anura species from Rize

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Abstract

The use of COI barcodes for specimen identification and species discovery has proven to be a functional molecular approach in the study of Anura. In this study, we evaluated the genetic status of *Bufo bufo*, *Hyla orientalis*, and *Rana macrocnemis* from different parts of Rize, Türkiye, using the COI gene marker, a popular method known as DNA barcoding. The Caucasus region is known to host a substantial amount of global biodiversity; however, limited barcoding studies are available for vertebrate taxa within this region. We have submitted all generated sequences to the BOLD data system, with the majority representing the first data from Rize in this database. Our analysis revealed four distinct barcode index numbers belonging to members of three different families. We observed a genetic distance of 0.038 between *B. bufo* specimens from İyidere and Çamlıhemşin. Within *R. macrocnemis*, genetic distances ranged from 0.018 to 0.020, and within *H. orientalis*, they ranged from 0.01 to 0.02. Our findings indicate that COI sequencing is a useful tool for the identification of anuran species present in Rize.

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Introduction

DNA barcoding is a genetic-based tool to identify and authenticate species using short DNA sequences. Different gene regions are used in DNA barcoding, however, the most frequently preferred gene region for animals is Cytochrome C Oxidase Subunit I (COI). Barcode of Life Database (BOLD) is a database that supports the recording, storing, and open publishing of barcoded DNA. In the DNA barcoding approach, DNA sequences of a specific, short and standardized genomic region are compared with a reference database (Savolainen et al. 2005; Hajibabaei et al. 2007). The standard genomic region used in DNA barcoding of animals is a 648-bp long fragment of the mitochondrial COI gene (Hebert et al. 2003). COI has proven highly efficient and reliable in many animal groups and is regularly used for various applications, such as for instance biodiversity assessments (Nagy et al. 2012; Hawlitschek et al. 2013).

The Caucasus region is among the most crucial global biodiversity hotspots, harboring rich endemic fauna and flora (Myers et al. 2000; Zazanashvili et al. 2004). Rize is one of the most spectacular provinces of the region in terms of biodiversity, due to its current climate characteristics, which include cool sum-

mers, mild winters, and rainy seasons throughout the year (Polat and Sunkar 2017). Some habitats are on the list of protected ecoregions declared by the IUCN (Heywood and Davis 1994), especially in the Kaçkar Mountains. Rize province hosts approximately 25% of amphibian species distributed throughout Türkiye. This study is the first to barcode the amphibians of Rize. DNA barcoding was applied to *Hyla orientalis*, *Bufo bufo*, and *Rana macrocnemis* from parts of Rize province.

During the study, we tested the universal amphibian primers, optimised reaction conditions, and sequenced the barcoding gene COI from various amphibian species, some of which were previously out of the focus of barcoding. We could identify the species or interspecific lineages based on the obtained sequences using bioinformatics and phylogenetic methods.

Materials and methods

We sampled 16 individuals from different localities in Rize (Türkiye) (Table 1). We obtained muscle tissue from individuals by toe-clipping during fieldwork and stored it in 95% alcohol. Total DNA was extracted from tissue samples using a Nucleospin DNA isolation kit (Macherey Nagel, Germany). We used a universal primer mix (comprising equal proportions of forward primers COI-C01 and COI-C02 and reverse primers COI-C03 and COI-C04) designed by Che et al. (2012) for polymerase chain reaction (PCR). Only COI-C01 and COI-C03 primers were used for sequencing. The following PCR protocol was used for amplification: initial denaturation for 5 min at 95°C; 36 cycles of denaturation for 1 min at 95°C, annealing for 1 min at 46°C, an extension for 1.15 min at 72°C; and final extension for 10 min at 72°C. PCR amplicons were confirmed by gel electrophoresis on a 0.8 % agarose gel containing ethidium bromide. We checked and edited the sequences via the software BioEdit (Hall, 1999). We submitted sequences to BOLD systems (Table 1). The operational taxonomic units (OTUs) were additionally inferred through Refined Single Linkage (RESL) analyses on BOLD (Ratnasingham and Hebert 2013). RESL was developed as a stepwise clustering process that uses single-link clustering as a tool for the preliminary assignment of records to an OTU. We used this method via the 'Cluster Sequences' tool available on the BOLD platform (<https://www.boldsystems.org>, accessed on 6 August 2024). We also assigned species through delimitation tests, such as Assemble Species by Automatic Partitioning (ASAP) (ASAP) (Puillandre et al. 2021) and Automatic Barcode Gap (ABGD) (Puillandre et al. 2012). ABGD was used to test for a barcode gap in the COI sequences of all amphibians (Relative gap = 1, Pmin=0.001, Pmax=0.1, Steps=10, NBins=10, K2P). ASAP is a recently developed method that infers the number of OTUs by implementing a hierarchical clustering algorithm based on pairwise genetic distances from single-locus sequence alignments (Puillandre et al. 2021).

Pairwise *p*-distances were used to calculate genetic divergences among species and genera with 500 replicates implemented in MEGA X (Kumar et al. 2018). We compared our sequences with data obtained from the BOLD system for each species. We calculated the number of haplotypes for each species and haplotype networks were reconstructed using Hapsolutely (Vences et al. 2024).

Table 1. Descriptive data for specimens from this study; N – Number of individuals.

Species	N	Locality	Haplotype	BOLD process ID	Base pair (bp)	Source
<i>Hyla orientalis</i>					580	
	5	City Center (Rize)	H1, H2	RZE009-23-RZE013-23		This study
	2	Lenkaran (Azerbaijan)	H3	AAE5180		BOLD
	1	Taşköprü (Kastamonu)	H4	AAE5180		BOLD
	3	Krym (Ukraine)	H5, H6,	AAE5180		BOLD
	1	Mediterranean	H7	AAE5180		BOLD
<i>Bufo bufo</i>					642	
	3	İyidere (Rize)	H1, H2, H3	RZE014-24-RZE016-24		This study
	4	Çat (Çamlıhemşin)	H1, H2, H3	RZE001-23-RZE004-23		This study
	1	Russia	H4	ABAAP009-08		BOLD
	1	Ukraine	H4	ABAAP045-08		BOLD
	8	Austria	H4, H5, H6, H7, H8	BCAHF044-17 BCAHF049-17 BCAHF050-17 BCAHF098-17 BCAHF127-19 BCAHF144-19 BCAHF167-19 BCAHF170-19		BOLD
	11	Germany (Bavaria)	H4, H9, H10, H11	FBHER016-09 FBHER017-09 FBHER042-09 FBHER043-09 FBHER044-09 FBHER050-09 FBHER052-09 FBHER059-09 FBHER060-09 FBHER084-09 FBHER085-09		BOLD
	3	Norway (Akershus)	H4, H13	HERNO009-18 HERNO010-18 HERNO011-18		BOLD
	1	Mediterranean	H12	GBAP20347-19		BOLD (Ehl et al. 2019)
	5	Russia	H14, H15	ABAAP001-08 ABAAP004-08 ABAAP005-08 ABAAP007-08 ABAAP042-08		BOLD
	4	Georgia	H16, H17	ABAAP146-08 ABAAP147-08 ABAAP148-08 ABAAP149-08		BOLD
<i>R. macrocnemis</i>					510	
	4	Çamlıhemşin (Rize)	H5, H6, H7	RZE005-23-RZE007-23		This study
	1	Kars (Susuz)	H1	ABAAP108-08		BOLD
	1	Hakkari (Beytuşebap)	H2, H3	ABAAP113-08 ABAAP115-08 ABAAP116-08 ABAAP117-08		BOLD
	1	Armenia (Garni)	H4	ABAAP122-08		BOLD

Results

Specimens of *Bufo* from Çat plateau (Kaçkar Mountains in Çamlıhemşin) and the İyidere (Rize) region were assigned different BIN numbers in the BOLD system (Tables 2 and 3), indicating the existence of two different lineages. Specimens from Çat were grouped with specimens from Georgia according to the BOLD identification system and identified as *B. verrucosissimus*. ASAP and ABGD tests assigned two OTUs for the *B. bufo* specimens from İyidere and Çat. For this lineage, we found a low genetic distance between specimens from Türkiye, Georgia, and Russia (Rize–Türkiye and Russia: 0.017; Türkiye and Georgia: 0.003). Haplotype network analysis revealed the same genetic relationships among localities (Fig. 1). The sequences of specimens from İyidere were assigned to *B. bufo* in the BOLD System. For this lineage, we found the mean genetic distance between specimens to be 0.038, ranging from 0.03 to 0.05. For this lineage, the genetic distance between specimens from İyidere (Rize, Türkiye) and the BOLD database (Ukraine, Russia, Austria, Germany, Norway) was 0.03 in all cases. According to haplotype network analysis, *B. bufo* specimens from European localities are genetically close to each other. However, researchers noted asymmetric mtDNA introgression in *B. bufo* species in Rize (Dursun et al. 2023). We found 17 haplotypes for the two mitochondrial lineages of *B. bufo* from our data and the BOLD system (both submitted as *B. bufo* and *B. verrucosissimus*). Specimens from İyidere and Çamlıhemşin were grouped in the same position on the haplotype network (Fig. 1A). Haplotypes of specimens from Georgia and Türkiye are situated close together. On the other hand, specimens submitted as *B. bufo* were grouped in haplotype network analysis (Table 1; Fig. 1).

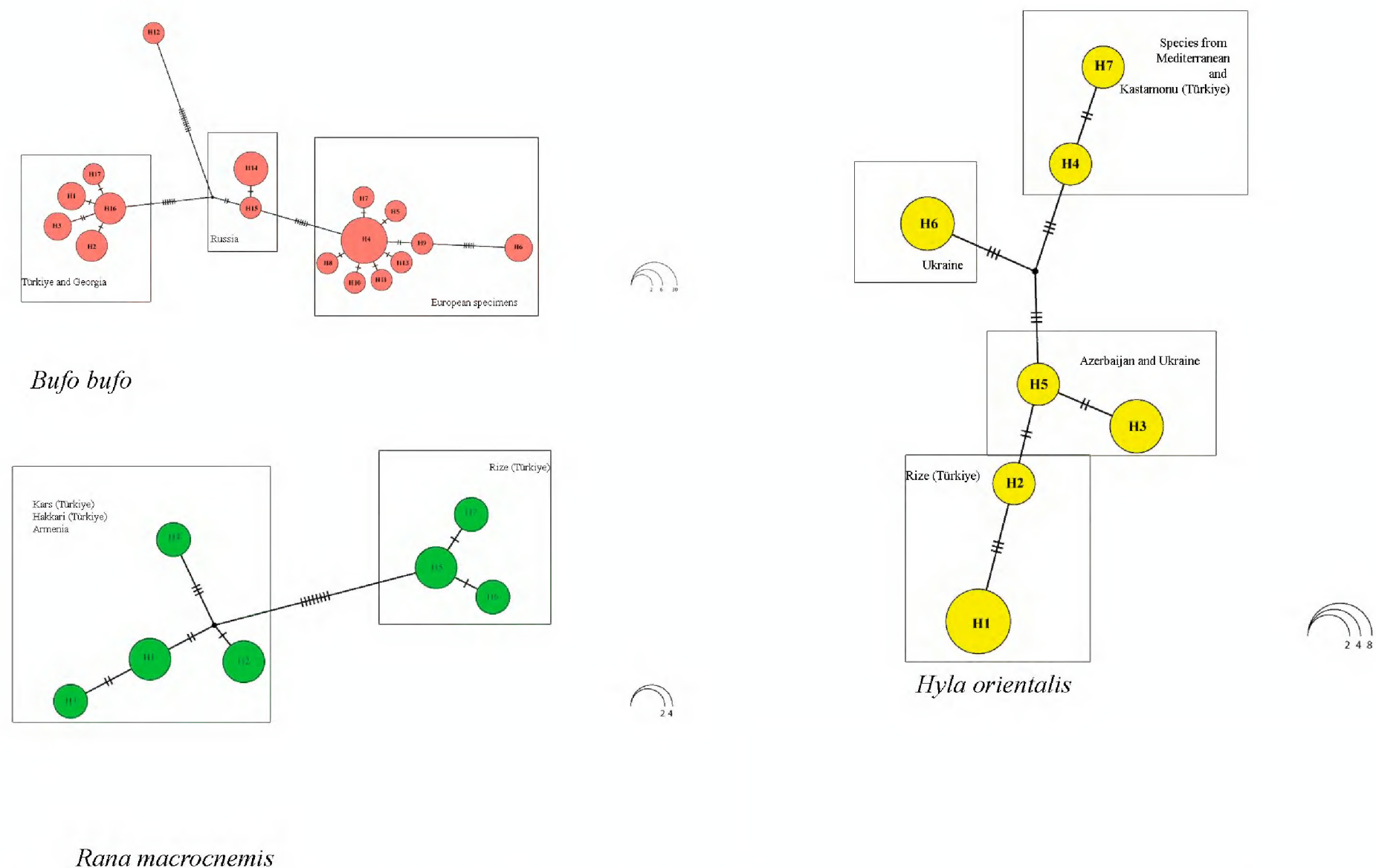
Rana macrocnemis – We found the genetic distance between Çamlıhemşin (Rize, Türkiye) and Kars (Türkiye) as 0.018, Hakkari (Türkiye) as 0.018, and Ar-

Table 2. Summary Report for Cluster Sequence Result from BOLD database

OTU	Mean Genetic Distance	Maximum Genetic Distance	Species
OTU-1	0.35	0.48	The mitochondrial lineage of <i>B. bufo</i> (Çamlıhemşin; Rize, Türkiye)
OTU-2	0.20	0.39	<i>R. macrocnemis</i>
OTU-3	0.76	1.90	<i>H. orientalis</i>
OTU-4	1.80	2.32	The mitochondrial lineage of <i>B. bufo</i> (İyidere; Rize, Türkiye)

Table 3. BIN number and *p* distances among specimens from Rize and data from BOLD

Species	BOLD BIN Number	<i>p</i> -distances
The mitochondrial lineage of <i>B. bufo</i> (İyidere; Rize, Türkiye)	ACE5152	0.017 (<i>B. verrucosissimus</i> , Russia); 0.03 (<i>B. verrucosissimus</i> , Georgia)
The mitochondrial lineage of <i>B. bufo</i> (Çamlıhemşin; Rize, Türkiye)	AAC2139	0.03 (<i>B. bufo</i> , Ukraine, Russia, Austria, Germany and Norway)
<i>H. orientalis</i>	AAE5180	0.01 (<i>H. orientalis</i> , Azerbaijan) 0.02 (<i>H. orientalis</i> , Kastamonu; Türkiye) 0.01 (<i>H. orientalis</i> , Ukraine)
<i>R. macrocnemis</i>	AFG1725	0.018 (<i>R. macrocnemis</i> , Hakkari and Kars) 0.02 (<i>R. macrocnemis</i> , Armenia)



Figures 1. Haplotype network of species from Rize and BOLD data specimens (Haplotype data is given in Table 1).

menia 0.020. According to haplotype network analysis, we also found low genetic differences (Fig. 1).

Hyla orientalis – Genetic distance was 0.01 between Azerbaijan and Rize (Türkiye) specimens, 0.02 between Kastamonu (Türkiye) and Rize (Türkiye) specimens, and 0.01 between Ukraine and Rize (Türkiye) specimens in *H. orientalis*. Specimens from Rize (Türkiye) are more similar to specimens from Ukraine and Azerbaijan than those from Kastamonu (Türkiye) (Fig. 1).

Despite RESL analysis being given four OTUs (Table 2), the other two discrimination tests (ASAP and ABGD) gave three OTUs for specimens (*H. orientalis*, *R. macrocnemis* and *B. bufo*) from Rize. We found the barcode gap distance to be 0.12 in the ABGD analysis. The sequence partition with the lowest ASAP score (1.5) distributed the sequences into 3 OTUs, and the threshold distance value was 0.116073.

Discussion

The Caucasus belongs to two (Caucasus and Irano-Anatolian) of the 36 global biodiversity hotspots (Myers et al. 2000; Mittermeier et al. 2011; Noss et al. 2015; Habel et al. 2020). The diversity of most animal taxonomic groups in this region is poorly understood, and a significant portion of the biodiversity data is outdated and incomplete (e.g., Mumladze et al. 2020). The Eastern Black Sea region is one of the most biologically rich regions in Türkiye, and Rize harbors a substantial part of this region's endemic fauna. This study represents the

first application of DNA barcoding to identify Rize's amphibian fauna. We also present the initial barcoding data for two mitochondrial lineages of *Bufo bufo* in the BOLD system from Türkiye.

Jablonski and Sadek (2019) asserted that populations from the Levant belong to *B. verrucosissimus*, closely affiliated with populations from the South Caucasian region rather than southern Türkiye, based on 16S rRNA. Dursun et al. (2023) estimated genetic differences between *B. bufo* specimens from Çamlıhemşin and İyidere as 0.023-0.026, according to the Cytochrome b (Cyt-b) gene region. We found a higher genetic distance using the COI gene region. More variable results were observed using mtDNA than nDNA in the *Bufo* species complex. Although we found moderate genetic differentiation between İyidere and Çamlıhemşin specimens, they appeared as the same haplotype in network analysis. Researchers have repeatedly noted that mtDNA oversplits species from hybrid zones in the *Bufo* species complex, attributed to asymmetric mtDNA introgression (Dursun et al. 2023).

Recuero et al. (2012) reported that the mean uncorrected pairwise genetic distances within *B. bufo* were 0.8% for combined 16S and Cyt-b data. We found the mean corrected distance ranged from 0.3-0.5% for two mitochondrial lineages of *B. bufo*. We determined that mitochondrial lineages of *B. bufo* from Rize, Türkiye, are genetically closer to Georgian specimens than to Russian, according to haplotype network analysis.

Genetic divergence of *Rana macrocnemis* is attributed to the last glaciation period, and gene flow among populations is restricted due to physical barriers and climate (Najibzadeh et al. 2017, 2021). Previously, genetic distances between *R. macrocnemis* populations were estimated as 0.002 and 0.016 within Türkiye (Kalaycı et al. 2017). In our case, the genetic distance was higher between the Çamlıhemşin (Rize, Türkiye) population and those from BOLD (Kars (Türkiye) (0.018), Hakkari (Türkiye) (0.018), Armenia (0.020), and an unknown locality from Türkiye (0.018)). We found no shared haplotypes among *R. macrocnemis* specimens in the Çamlıhemşin population.

Although we found low genetic differences among specimens from Azerbaijan, Kastamonu (Türkiye), Ukraine, and Rize (Türkiye) in *Hyla orientalis*, we found no shared haplotypes among localities. This species showed high genetic differentiation using nuclear markers in the Caucasian region (Birbele et al. 2023), while mtDNA usually yields lower divergence. Unusual environmental factors induced genetic variability in the *H. orientalis* population inhabiting the Chornobyl exclusion zone in Ukraine (Car et al. 2022). This could explain the discordance between mtDNA and nDNA differences in *H. orientalis* in this region.

Different species delimitation tests yielded somewhat different results for the classification of OTUs. For example, RESL gave four OTUs for the specimens from Rize (including two lineages of *Bufo* – resembling *B. bufo* and *B. verrucosissimus* as given in BOLD), while in contrast, ABGD and ASAP gave three OTUs (Table 2). In general, ASAP and ABGD were more conservative than RESL analysis (Macko et al. 2024). Nevertheless, the barcode data in BOLD databases are still incomplete (limited coverage of barcode variation or lacking certain species entirely), therefore accurate identification may be hindered. Interpreting the results can also be challenging (Abdi et al. 2024).

Researchers strongly recommend more local efforts to enrich the global list of barcoded species (Drohvalenko et al. 2019; Weigand et al. 2019). This study is the first to reveal the genetic diversity of Anura from the Rize region and to improve data accuracy in the universal database (BOLD). DNA barcoding is a vital tool for biodiversity research and conservation plans, offering promise for rapid species identification and biodiversity assessments in hotspot areas. We further encourage the use of DNA barcoding to monitor genetic diversity and resources across a wider geographic range and in specimens throughout Türkiye.

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Additional information

Conflict of interest

The authors have declared that no competing interests exist.

Ethical statement

No ethical statement was reported.

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Author contributions

BG - field and laboratory work, data analyses,, writing, review; TEK - study design, supervising, writing original draft.

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Data availability

All of the data that support the findings of this study are available in the main text or Supplementary Information.

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